Adenophostin A Induces Spatially Restricted Calcium Signaling in *Xenopus laevis* Oocytes*

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The activation of intracellular calcium release and calcium entry across the plasmalemma in response to intracellular application of inositol 2,4,5-trisphosphate and adenophostin A, two metabolically stable agonists for inositol 1,4,5-trisphosphate receptors, was investigated using *Xenopus laevis* oocytes and confocal imaging. Intracellular injection of inositol 2,4,5-trisphosphate induced a rapidly spreading calcium signal associated with regenerative calcium waves; the calcium signal filled the peripheral regions of the cell in 1–5 min. Injection of high concentrations of adenophostin A (250 nM) similarly induced rapidly spreading calcium signals. Injection of low concentrations of adenophostin A resulted in calcium signals that spread slowly (>1 h). With extremely low concentrations of adenophostin A (<10 nM), stable regions of Ca\(^{2+}\) release were observed that did not expand to peripheral regions. When the adenophostin A-induced calcium signal was restricted to central regions, compartmentalized calcium oscillations were sometimes observed. Restoration of extracellular calcium caused a rise in cytoplasmic calcium that did not expand to peripheral regions. When the adenophostin A-induced calcium signal was restricted to central regions, compartmentalized calcium oscillations were sometimes observed. Restoration of extracellular calcium caused a rise in cytoplasmic calcium that did not expand to peripheral regions. When the adenophostin A-induced calcium signal was restricted to central regions, compartmentalized calcium oscillations were sometimes observed. Restoration of extracellular calcium caused a rise in cytoplasmic calcium that did not expand to peripheral regions.

*Xenopus laevis* oocytes have proven a useful model system for studying the inositol 1,4,5-trisphosphate (1,4,5)IP\(_3\)-mediated calcium signaling system, providing insights into mechanisms of calcium oscillations (1) and capacitative Ca\(^{2+}\) entry (2, 3). In many cell types, including *Xenopus* oocytes, activation of surface membrane receptors results in a biphasic Ca\(^{2+}\) response composed of an initial mobilization of intracellular Ca\(^{2+}\), followed by entry of extracellular Ca\(^{2+}\) (4). Much is known about the role of (1,4,5)IP\(_3\) in mobilizing intracellular Ca\(^{2+}\), and a working model for the Ca\(^{2+}\) entry process is described by the capacitative model (5, 6) according to which the depletion of intracellular (1,4,5)IP\(_3\)-sensitive Ca\(^{2+}\) stores signals the activation of plasma membrane calcium channels.

Recently, a metabolite of *Penicillium brevicompactum*, adenophostin A, has been isolated and demonstrated to be an agonist for the (1,4,5)IP\(_3\) receptor and to have a potency ~100-fold greater than that of (1,4,5)IP\(_3\) (7). Interestingly, two studies performed in *Xenopus* oocytes (8, 9) suggested a site of action for adenophostin A in addition to that on intracellular Ca\(^{2+}\) stores. In both studies, low concentrations of adenophostin A (<10 nM) appeared to preferentially activate the Ca\(^{2+}\) entry process, although Hartzell et al. (9), unlike DeLisle et al. (8), could not find a clear dissociation between Ca\(^{2+}\) release and Ca\(^{2+}\) entry. In a subsequent report, Machaca and Hartzell (10) found that the diffusion of adenophostin A throughout the oocyte was considerably slower than that of IP\(_3\) and concluded that this might explain the apparently diminished Ca\(^{2+}\) release signal.

In this study, we have used confocal microscopy to monitor directly spatial as well as temporal aspects of the effects of adenophostin A on intracellular Ca\(^{2+}\) in *Xenopus* oocytes using the calcium-sensitive dye Calcium Green. Our results confirm that diffusion of adenophostin A throughout the oocyte is slower than that of IP\(_3\) and additionally indicate that low concentrations of adenophostin A cause a confined mobilization of intracellular Ca\(^{2+}\) that is capable of supporting spatially restricted Ca\(^{2+}\) oscillations and spatially restricted Ca\(^{2+}\) entry. This unique action of adenophostin A provides new insights into the role of IP\(_3\) receptor binding in Ca\(^{2+}\) oscillations and also into the spatial relationships between intracellular Ca\(^{2+}\) release and activation of capacitative calcium entry.

**MATERIALS AND METHODS**

**Isolation of Xenopus Oocytes**

Adult albino female *X. laevis* (Xenopus One, Ann Arbor, MI) were anesthetized by hypothermia and decapitated. The ovaries were then removed and stored in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 5 mM HEPES (pH 7.6)), Stage V and VI oocytes were manually dissected free from the ovaries and follicles and maintained in ND96 buffer at 18 °C until used.

**Oocyte Microinjection**

Various solutions (described below) were injected into oocytes via an oil-filled glass micropipette attached to a nanoliter injector (World Precision Instruments, Inc., Sarasota, FL). The injection volume could be varied from 4.6 to 50.6 nl, and final concentrations were calculated on the basis of an oocyte volume of 1 μl.

**Calcium Measurement**

**Calcium Green Loading**—Prior to the experiment, defolliculated oocytes were microinjected with the Ca\(^{2+}\) indicator Calcium Green (~12.5 μM final concentration; Molecular Probes, Inc., Eugene OR). The oocytes were then incubated for at least 30–40 min at room temperature to allow equilibration of the dye throughout the cytoplasm. Oocytes were placed in a glass-bottomed microscope chamber (Bionique Testing Laboratories, Inc.) containing 1 ml of ND96 buffer and placed on the stage of an inverted Zeiss confocal microscope (LSM 410, Carl Zeiss Inc., Thornwood, NY). Oocytes were held in position by a microinjection...
pipette, and modifications of the bathing solution were made by dilution into the bath.

**Fluorescence Measurements**—Measurements of intracellular calcium were performed with the inverted Zeiss LSM 410 confocal microscope equipped with a 10× objective (0.5 numerical aperture). The calcium-sensitive dye Calcium Green was excited by the 488-nm line from a krypton-argon laser (Omnimicroscope Model 643, Melles Griot, Carlsbad, CA), and the emission fluorescence monitored at 515 nm was selected by a band-pass filter. The pinhole aperture of the confocal microscope was set such that the fluorescence image represented an optical slice of \( \sim 10 \mu m \) (if not otherwise specified) at the bottom of the oocyte, *i.e.* just above the coverslip and just under the plasma membrane (Fig. 1). All fluorescence images (256 intensity levels, 256 × 256 pixels) were detected with an analog photomultiplier tube, and the resulting images were recorded directly onto videotape. Simultaneously, up to 10 regions of interest (ROIs) distributed across the image provided an intensity versus time graphic output. Subsequently, these fluorescence values were expressed as a fraction of the initial fluorescence intensity (F/F_0).

During fluorescence data collection, each scan of a 256 × 256 image took 0.35 s, and the interval between each image scan was \( \sim 1.3 \) s.

**Photolysis of Caged Compounds**—In some experiments, oocytes were co-injected with caged (1,4,5)IP_3 (5–10 \( \mu m \) final concentration; Calbiochem) along with the calcium dye. Photocleavage of caged (1,4,5)IP_3 was achieved using the 351/364-nm lines of an argon ion laser (Coherent Enterprise Model 652, Coherent Inc., Santa Clara, CA), liberating (1,4,5)IP_3 into the cytoplasm. The area in which photocleavage occurred was defined by use of a fast gated Uniblitz shutter (Vincent Associates, Rochester, NY) placed between the argon ion laser and the confocal microscope. By varying the duration the shutter was open (typically 5–10 ms), the area of photolysis could be varied in the xy plane.

**RESULTS**

In earlier studies (8–10), the actions of adenophostin A were compared with those of the physiological agonist for the IP_3 receptor, (1,4,5)IP_3. However, adenophostin A differs from (1,4,5)IP_3 in being resistant to inactivation by the 5-phosphatase and 3-kinase (7). Thus, for the majority of our studies, we chose to utilize a metabolism-resistant analog of (1,4,5)IP_3, (2,4,5)IP_3 (11), for comparison with adenophostin A. Fig. 2 shows the pattern of [Ca^{2+}], signaling seen following injection of (2,4,5)IP_3 at a concentration of 1 \( \mu m \) (final estimated cytoplasmic concentration). This somewhat intermediate concentration of (2,4,5)IP_3 caused a spreading [Ca^{2+}] signal that filled the field of view generally within 5 min. At this concentration, the [Ca^{2+}] signal was usually associated with regenerative waves and spirals, as originally described by Lechleiter *et al.* (1).

Also shown in Fig. 2 is a typical arrangement of ROIs used for assessing the time course of [Ca^{2+}] changes in the central and peripheral regions of the field of view. Fig. 3 illustrates examples of the time course of [Ca^{2+}] changes in the peripheral and central regions of oocytes following the injection of four different concentrations of (2,4,5)IP_3. In these experiments, the oocytes were initially incubated in a Ca^{2+}-deficient medium, and extracellular Ca^{2+} was subsequently restored to 10 \( \mu m \) to assess [Ca^{2+}] signals due to influx. At the highest concentration (5 \( \mu m \)), diffusion throughout the oocyte was very rapid such that a rapid release occurred in the peripheral regions of the field of view seconds after appearing in the center. With 1 \( \mu m \) (2,4,5)IP_3, a somewhat greater delay was observed before the peripheral regions were activated, and as shown in Fig. 2, regenerative [Ca^{2+}] oscillations were seen. With both 5 and 1 \( \mu m \) (2,4,5)IP_3, re-addition of Ca^{2+} externally resulted in a rapid increase in [Ca^{2+}] indicative of Ca^{2+} entry, presumably due to the depletion of intracellular stores. With lower concentrations of (2,4,5)IP_3 (250 and 100 \( \mu m \)), release was seen only near the center of the field of view, and with these concentrations, the signal was seen only when the injection pipette was brought deep into the oocyte, close to the coverslip and the field of view. These smaller signals did not propagate to the periphery, and restoration of extracellular Ca^{2+} did not result in the activation of significant entry. The simplest interpretation of this result is that the lower concentrations of (2,4,5)IP_3 are insufficient to activate significant Ca^{2+} mobilization when finally diluted by diffusion throughout the cytoplasm. This is as not unexpected since the \( K_d \) for (2,4,5)IP_3...
action on the IP₃ receptor is >1 μM (12).

A noticeable different picture was seen with adenophostin A. Fig. 4 illustrates a series of images depicting the spread of the [Ca²⁺] signal in an oocyte injected with adenophostin A to give a final concentration of 10 nM. This concentration of adenophostin A should, if anything, be somewhat more potent than 1 μM (2,4,5)IP₃, yet between 30 and 60 min was required for the [Ca²⁺] signal to fill the field of view. The full time courses of four peripheral and four central ROIs from this experiment and the experiment shown in Fig. 2 are given in Fig. 5.

Although somewhat larger than (2,4,5)IP₃, adenophostin A would not be expected to diffuse at such a relatively slow rate unless some structure specifically impedes its rate of diffusion. It seems likely that this structure is the IP₃ receptor itself. Thus, the concentrations of adenophostin A injected into the oocytes may be lower than the concentration of IP₃ receptors, and when adenophostin A molecules bind to these receptors with high affinity, their rate of diffusion through the cell is dependent to a large extent on the rather slow rate of receptor dissociation.² A prediction then is that higher concentrations of adenophostin A would diffuse much faster throughout the cell. Fig. 6 shows that with concentrations of 250 and 100 nM adenophostin A, the [Ca²⁺] signal reached the peripheral regions of the field of view much faster than with 10 nM (within 1 min or so). As for (2,4,5)IP₃, once the signal had filled the field of view, restoration of extracellular Ca²⁺ resulted in rapid influx of Ca²⁺. Fig. 7 summarizes the data on the influx of Ca²⁺ in oocytes activated by relatively high concentrations of (2,4,5)IP₃ (10 μM) and adenophostin A (250 nM). Under these conditions, the magnitude of the influx and its sensitivity to extracellular Ca²⁺ were indistinguishable with these two IP₃ receptor ligands.

We next investigated the effects of injecting very low concentrations of adenophostin A into oocytes. When injections were carried out with quantities of adenophostin A that would give cytoplasmic concentrations of ~10 μM, we saw no changes in [Ca²⁺]. However, when the pipette was brought very close to the coverslip and the field of view, a localized area of elevated [Ca²⁺] was observed. In contrast to the findings with low concentrations of (2,4,5)IP₃, this elevated region was relatively stable; the size of the region did not grow appreciably, and the elevated [Ca²⁺] level was maintained for up to 30 min. In some oocytes, [Ca²⁺] waves and spirals developed within this region (Fig. 8). These waves were similar in appearance to those seen in oocytes injected with 1 μM (2,4,5)IP₃, but the waves always extinguished when reaching the limiting periphery of the region of activation. The average wave velocities were 27.3 ± 2.5 μm/s (n = 3) for (2,4,5)IP₃ and 8.5 ± 0.6 μm/s (n = 7) for adenophostin A. The value for (2,4,5)IP₃ is within and that for adenophostin A is just below the range reported for agonist-dependent waves first described by Lechleiter et al. (1). Since wave velocity is dependent on the diffusional distance between release sites, the lower value for adenophostin A may indicate that the concentration of adenophostin A-bound receptors is lower than for (2,4,5)IP₃. We presume that the limited size of this activated region is determined by the limits of diffusion and action of adenophostin A. Thus, the restricted area of [Ca²⁺] waves underscores the absolute requirement for activated IP₃ receptors for propagation to continue. The presence of such waves under conditions whereby adenophostin appears essentially irreversibly affixed to the IP₃ receptor indicates that the affinity of the ligand for the IP₃ receptor, and specifically the rate of dissociation of the ligand from the IP₃ receptor, is not a determinant of wave generation or propagation.

We examined the activation of Ca²⁺ entry in oocytes injected with low concentrations of adenophostin A. Fig. 9 illustrates a result with an oocyte injected with 10 nM adenophostin A. With this concentration, spread of [Ca²⁺], was slow, but eventually filled the entire cell. Addition of external Ca²⁺ at a time when [Ca²⁺] was elevated in the central regions, but not yet in the peripheral regions, of the cell resulted in a further rise in [Ca²⁺] in the central regions, but no increase in the periphery. Thus, these localized responses to adenophostin A are apparently capable of activating Ca²⁺ entry.

There is considerable evidence that the endoplasmic reticulum of cells is at least somewhat continuous throughout the cell (13). Thus, we considered the possibility that despite the restricted localization of the sites of Ca²⁺ release within the oocyte, Ca²⁺ may become depleted from the lumen of the endoplasmic reticulum in the cellular periphery and that this depleted endoplasmic reticulum may contribute to the signaling of Ca²⁺ entry. To investigate this possibility, we injected an oocyte with caged (1,4,5)IP₃ along with Calcium Green. In the experiment shown in Fig. 10, after insertion of the microinjection pipette, the oocyte was briefly (5 s) exposed to the UV laser, liberating (1,4,5)IP₃ in a small area of the cytoplasm (in

²Machaca and Hartzell (10) came to a somewhat similar conclusion based on a reduction of the effective concentration of adenophostin A as a result of its binding to IP₃ receptors.
This resulted in a rapid and transient release of intracellular Ca\(^{2+}\). Subsequently, the same oocyte was injected with adenophostin A to give a final concentration (if fully diluted) of \(10^{-10}\) pM. This quantity of adenophostin A gave a relatively stable localized region of elevated \([\text{Ca}^{2+}]_i\). Exposure to the UV laser revealed that the release of Ca\(^{2+}\) in the peripheral regions appeared similar to that seen prior to adenophostin A injection. Following the discharge of Ca\(^{2+}\) by the photolytic release of (1,4,5)IP\(_3\), the liberated Ca\(^{2+}\) was re-accumulated presumably following the time course of diffusion and metabolism of (1,4,5)IP\(_3\). This indicates that when the action of adenophostin A is restricted to specific cellular regions, the endoplasmic reticulum Ca\(^{2+}\) stores outside of that region remain fully charged and functional.
Fig. 11 illustrates an experiment in which Ca$^{2+}$ entry was assessed in an oocyte injected with the lowest concentration of adenophostin A used (10 pM). In this particular cell, an initial central release of Ca$^{2+}$ was followed by the appearance of [Ca$^{2+}$]i oscillations. Addition of 10 mM extracellular Ca$^{2+}$ resulted in a sustained rise in [Ca$^{2+}$]i restricted to the region of the cell influenced by the adenophostin A injection. Elevation in [Ca$^{2+}$]i was seen in the cell periphery only in response to liberation of (1,4,5)IP$_3$ from its caged precursor.

**DISCUSSION**

The results of this study demonstrate that adenophostin A has the unique ability to activate and regulate [Ca$^{2+}$]i signaling in precise, spatially restricted regions of the cell. The reason for this behavior is likely due to the high affinity of adenophostin A for the IP$_3$ receptor and its presumed slow rate of dissociation from the receptor. In a previous report on the binding of IP$_3$ to its receptor, Spaet et al. (12) pointed out that the (1,4,5)IP$_3$ signaling system worked efficiently because the concentration of cellular receptors was less than the $K_d$ of (1,4,5)IP$_3$ for its receptor. When the concentration of receptor is higher than the $K_d$ of its ligand, nonlinear behavior can result, and the receptor becomes a reservoir for ligand, reducing its free concentration (14). This is likely the situation with low concentrations of adenophostin A, although adenophostin A at higher concentrations functions as a full and potent activator of the (1,4,5)IP$_3$ pathway (15, 16).

Utilizing Ca$^{2+}$-dependent chloride currents as an indirect assay of [Ca$^{2+}$]i changes, both DeLisle et al. (8) and Hartzell et al. (9) observed activation of Ca$^{2+}$ entry with low concentrations of adenophostin A (<100 pM (8) and <50 pM (9)). DeLisle et al. saw no evidence of Ca$^{2+}$ release with these concentrations, whereas Hartzell et al. observed small chloride currents attributable to intracellular release. Both groups of investigators concluded that an IP$_3$ receptor was involved in the action of adenophostin A, possibly through releasing from a small subset of the intracellular stores. In a more recent publication,
The cellular medium was switched to one containing 10 mM calcium. This had occurred in the central (but not the peripheral) regions, the extracellular calcium from the central regions. After a significant rise in intracellular calcium in Figs. 4 and 5, the intracellular calcium changes developed slowly from the central regions. After a significant rise in intracellular calcium had occurred in the central (but not the peripheral) regions, the extracellular medium was switched to one containing 10 mM calcium. This increase in extracellular calcium had an immediate effect on the central regions where intracellular calcium release had already occurred, whereas significant changes in the peripheral regions occurred after a long delay (>10 min in this example). These data are typical of five different experiments.

Machaca and Hartzell (10) also observed that the spread of adenophostin A-induced [Ca\(^{2+}\)] signals was somewhat slower than for (1,4,5)IP\(_3\) and concluded that the slow release might diminish the magnitude of the associated chloride current. Our findings suggest an alternative explanation for the inability of adenophostin A to efficiently activate chloride currents associated with release. When chloride current is used as a measure of [Ca\(^{2+}\)], only changes in [Ca\(^{2+}\)] immediately under the plasma membrane can be observed. Clearly, any influx of Ca\(^{2+}\) must occur across the plasma membrane, and thus, the use of a plasma membrane-associated marker, such as Ca\(^{2+}\)-dependent chloride channels, will be a sensitive indicator of this mode of Ca\(^{2+}\) signaling. From the current results, it is clear that injections of small quantities of adenophostin A can induce a substantial degree of intracellular release of Ca\(^{2+}\), but that this release can occur at some distance from the plasma membrane and thus would not be seen by plasma membrane chloride channels. The opacity of the ooplasm, due to high concentrations of yolk proteins, makes it difficult to image deeply into the oocyte. Thus, to observe this spatially restricted action of adenophostin A, we injected close to the plasma membrane just above the coverslip. These regions of release presumably make contact with the plasma membrane where it sits on the coverslip. The Ca\(^{2+}\) influx observed in this situation may be derived from Ca\(^{2+}\) in the limited aqueous space between the oocyte and coverslip. Presumably with injections in more central regions of the cell, such as in the previous electrophysiological experiments, it would be possible to activate regions of Ca\(^{2+}\) release that make minimal contact with the plasma membrane of the oocyte. Such spatially restricted regions of Ca\(^{2+}\) depletion might signal to the plasma membrane to activate entry that would be detected as chloride current. In any event, it is obvious from the unusual behavior of adenophostin A that assessment of [Ca\(^{2+}\)] changes near the plasma membrane may severely underestimate the extent of cellular Ca\(^{2+}\) discharge by this agent, and this may result in the apparent dissociation between release and entry observed in earlier studies.

In a recent report, the activity of a putative diffusible calcium influx factor, CIF, was described (17). This material was produced by many cells, including cardiac myocytes and smooth muscle cells, and is known to be present in the extracellular space. CIF is a calcium-dependent protein that binds to the plasma membrane and activates calcium influx channels. The exact mechanism by which CIF activates calcium influx channels is not fully understood, but it is thought to involve a direct interaction between CIF and calcium influx channels. CIF is also known to be involved in the regulation of intracellular calcium levels, and its activity is thought to be important in the control of cell function.
capable of diffusing through and activating calcium influx in regions associated with intact endoplasmic reticulum stores. In the current studies, if such a factor had been released from the regions of adenophostin A action, it would be expected to diffuse into and activate entry in the peripheral regions of the oocyte; however, no such signal was observed. Thus, the coupling of intracellular calcium release to calcium entry in the oocyte appears to depend more intimately on the proximity of IP3-depleted calcium stores, perhaps because of the involvement of IP3 receptors in conveying the signal to the plasma membrane (18).

Although adenophostin A may not have unique actions on the calcium entry pathway, its unusual kinetic behavior affords an opportunity to control experimentally the extent of Ca2+ signaling spatially within a single cell. For example, the spatially restricted calcium waves, such as those shown in Fig. 8, make it clear that wave propagation cannot extend beyond the region of action of an IP3 receptor agonist. Since regenerative waves were frequently observed within this restricted region, it is clear that agonist affinity, and specifically the dissociation rate from the receptor, is not a factor in the propagation mechanism. This is somewhat surprising given the well documented inhibitory effect of Ca2+ on IP3 receptor affinity and specifically the dissociation rate (19, 20). This unusual ability of adenophostin A to produce spatially restricted regions of activated calcium signaling may prove useful in other ways in future studies of the spatial and temporal aspects of Ca2+ signaling.

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